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## CHARACTERIZATION AND AMINO ACID SEQUENCES OF TWO LETHAL PEPTIDES ISOLATED FROM VENOM OF WAGLER'S PIT VIPER, *TRIMERESURUS WAGLERI*

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S. A. WEINSTEIN, J. J. SCHMIDT, A. W. BERNHEIMER and L. A. SMITH. Characterization and amino acid sequences of two lethal peptides isolated from venom of Wagler's pit viper, *Trimeresurus wagleri*. *Toxicon* 29, 227-236, 1991.—Two lethal toxins were isolated from *Trimeresurus wagleri* venom by fast protein liquid chromatography (molecular sieve) and high performance liquid chromatography (reverse phase). The toxins (termed peptide I and II) had mol. wt of 2504 and 2530, respectively, pIs of 9.6-9.9 and lacked phospholipase A, proteolytic, and hemolytic activity. Lethal peptide I had a murine i.p. LD<sub>50</sub> of 0.369 mg/kg, while lethal II had a murine i.p. LD<sub>50</sub> of 0.583 mg/kg. Peptide I retained full toxicity after autoclaving at 121°C for 40 min. The lethal activity was found to represent less than 1% of the total venom protein, which was only 62-65% of crude venom. The amino acid sequence of peptide I revealed a proline-rich (over 30% of total sequence) sequence unique among snake venom toxins. Lethal peptide II showed the same sequence except for a second tyrosine in the position of histidine (residue No. 10) in peptide I. The toxin lacked antigenic identity with a number of representative neurotoxins and myotoxins. The crude venom shared at least one antigen with *Crotalus scutulatus scutulatus* venom. This antigen was not Mojave toxin. The toxin appears symptomologically suggestive of a vasoactive peptide or neurotoxin.

### INTRODUCTION

WAGLER'S pit viper *Trimeresurus* (= *Tropidolaemus*, BRATTSTROM, 1964) *wagleri* (LEVITON, 1964) is a small to medium-sized (65-70 cm average) arboreal crotaline which ranges throughout Malaysia, the Philippines, Thailand and the Indo-Australian archipelago to Indonesia. *T. wagleri* secretes one of the more toxic *Trimeresurus* venoms. A neurological site of action for *T. wagleri* venom was proposed by SMITH and HINDLE (1931). MINTON (1968) described a component of *T. wagleri* venom which was dialyzable (smaller than 1000 mol. wt), and remarkably thermostable. TAN and TAN (1989) used

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Sephadex G-50 gel filtration and SP-Sephadex C-25 ion exchange to obtain two non-enzymatic toxins. Gel filtration indicated an  $M_r$  of 8900. These workers found that crude *T. wagleri* venom did not affect nerve-evoked twitch tension or acetylcholine reduced response of chick biventer muscle, thus suggesting that *T. wagleri* venom toxins were not neurotoxins. The present study describes purification, characterization and amino acid sequences of two lethal toxins from the venom of *T. wagleri*.

## MATERIALS AND METHODS

### Chemicals

Trifluoroacetic acid (TFA), "sequenal grade", was purchased from Pierce Chemical Company, Rockford, IL. Acetonitrile and water ("HPLC grade") were from Thomas Scientific, Swedesboro, NJ, while 4-vinylpyridine was from Sigma Chemical Co., St Louis, MO.

### Venoms and purified toxins

*Trimeresurus wagleri* venom was obtained from Ventoxin Laboratories (Frederick, MD), *Naja naja kaouthia* venom from Biotoxins, Inc. (St Cloud, FL), and *Oxyuranus scutellatus scutellatus* venom from Venom Supplies (Tanunda, South Australia). The *T. wagleri* venom was collected as a pool from three young adult specimens (all females). Individual samples were collected from each specimen and examined separately in order to establish lack of venom protein variation among these snakes. *Androctonus australis* venom was purchased from Latoxan (Rosans, France).

Crotamine (lot 17) was purchased from Miami Serpentarium Laboratories, Salt Lake City, UT.  $\alpha$ -Cobratoxin was purified from venom of *Naja naja kaouthia* by using Sulphopropyl (SP)-Sephadex chromatography (CHATMAN and DiMARI, 1974). Taipoxin was purified from venom of *Oxyuranus s. scutellatus* by gel filtration (FOHLMAN *et al.*, 1976). Mojave toxin was purified from venom of *Crotalus scutulatus scutulatus* (a single adult male from Yuma, AZ) according to the method of WEINSTEIN *et al.* (1985). Toxin II from *Androctonus australis* venom (AaH II) was purified by successive fractionation on Mono Q, Mono S and Superose-12 column chromatography (Pharmacia, Piscataway, NJ). Purity and identity of crotamine,  $\alpha$ -cobratoxin, taipoxin and AaH II were determined by reverse-phase chromatography and amino acid sequence analysis.

### Antibodies

Antisera against purified toxins were raised in New Zealand white rabbits using an administration schedule essentially as described by KAISER *et al.*, (1986) with the exception that different toxin dosages were used in the immunization protocol. Crotamine was administered at 10  $\mu$ g/kg rabbit,  $\alpha$ -cobratoxin and AaH II were administered at 6  $\mu$ g/kg rabbit, and taipoxin was used at 2  $\mu$ g/kg rabbit. When antibody titers were detected, doses were increased by 25%. IgG was purified from serum using DEAE-Affi-Gel Blue (Bio-Rad, Richmond, CA). Polyclonal antibody against crude *C. scutulatus scutulatus* venom (Type A) and purified Mojave toxin was prepared following the immunization schedule of WEINSTEIN *et al.* (1985).

### Lethality determinations

The i.p. LD<sub>50</sub> of the crude venom was obtained by injection of male Swiss-Webster mice (18–20 g) in five groups of four mice per group. All injections were administered in the lower quadrants of the abdomen. Dosage was derived from a 1 mg/ml solution of venom in phosphate buffered saline (0.05 M, pH 7.2). Animals were observed after injection and mortality recorded after 24 hr. The LD<sub>50</sub> of lethal fractions from Superose-12 (molecular sieve) chromatography was determined by injecting male Swiss-Webster mice (18–20 g) in five groups which contained four mice per group, while the lethal potency of HPLC fractions was determined using five groups which consisted of four mice per group. Toxin diluent was Tris-HCl (0.05 M, pH 7.2) containing 0.7 M NaCl. In the first two dose levels involving venom fractions, equivalent volumes of buffer were injected into control groups of two mice per group. Animals succumbing to either crude venom or venom fraction dosages were necropsied and any gross tissue pathology examined and noted. The LD<sub>50</sub> was calculated by the Spearman-Kärber method (WHO, 1981). The 95% fiducial limits for the LD<sub>50</sub> were determined.

### Determination of protein concentration

Protein concentrations were estimated from the molar extinction coefficients of the two purified peptides. The bicinchoninic acid assay (BCA assay, Pierce Chemicals) (SMITH *et al.*, 1985) was used only as a comparative method to determine the accuracy with these peptides of standard protein assay methods.

#### *Venom fractionation*

Venom samples were prepared for chromatographic analysis by dissolving 18–28 mg in 1 ml of 0.05 M Tris HCl, pH 7.2 containing 0.7 M NaCl. Aliquots of 500  $\mu$ l were centrifuged at 100,000  $\times$  g in an Eppendorf 5415 benchtop centrifuge (Eppendorf Laboratories), and the supernatants were chromatographed on a 1.0  $\times$  30 cm Superose-12 column (Pharmacia LKB Biotechnology, Piscataway, NJ). Fractions which exhibited lethality in mice were acidified with TFA and subjected to reverse phase chromatography (Waters Associates, Milford, MA) using a Hi-Pore RP-318 column (Bio-Rad Laboratories, Richmond, CA). Fractions obtained from reverse phase chromatography were neutralized with N-ethylmorpholine (1.0–1.5  $\mu$ l/ml of fraction) and most of the acetonitrile was removed by evaporation with a stream of dry nitrogen at room temperature. Fractions were then screened in mice for lethality. Control animals were injected with neutralized TFA only.

#### *Sequence analyses*

Prior to sequencing, samples were reduced and pyridylethylated as described previously (CAVINS and FRIEDMAN, 1970; SCHMIDT and MIDDLEBROOK, 1989). Automated Edman degradation was then done in a model 470A amino acid sequencer from Applied Biosystems, Foster City, CA. Residues were identified with an on-line high pressure liquid chromatograph (model 120A, from the same manufacturer).

#### *Amino acid compositions*

Samples were dried in heavy-wall ignition tubes, then redissolved in 0.70 ml 6N HCl/0.002 M phenol. Tubes were sealed under vacuum and placed in a heating block at 110°C for 30 hr. The amino acid compositions of the hydrolysates were determined with a Beckman System 7300 amino acid analyzer (Beckman Instruments, Palo Alto, CA).

#### *Isoelectric focusing (IEF)*

IEF was performed in a 110 ml electrofocusing column (LKB Instruments). The gradient was prepared from a less dense solution consisting of 51 ml of water, 4 ml of 8% (w/v) ampholine (pH 3–10) and a more dense solution consisting of undialyzed sample, 8.5 ml of 8% (w/v) ampholine (pH 3–10) and 25 g of sucrose in a final volume of 55 ml. Focusing was carried out at 4°C for 24 hr with a final potential of 1000 V. Fraction volume was 4 ml.

#### *Determination of proteolytic activity*

Presence of proteolytic activity was determined by use of a casein agarose gel substrate (Bio-Rad Laboratories). Venom solutions from 0.125 mg/ml to 1 mg/ml were added to wells punched in the gel plate, and zones of clearing were measured after 20 hr at 25°C. A 1 mg/ml trypsin solution similarly prepared was used as a standard. PBS (0.05 M, pH 7.2) was used as diluent for venom and trypsin solutions. A well filled with PBS served as a negative control.

#### *Elisa*

Enzyme-linked immunosorbent assays (ELISA) were carried out essentially as described by HENDERSON and BIEBER (1986). Peroxidase-conjugated, goat anti-rabbit IgG (Sigma) served as secondary antibody when the primary antibody used was rabbit polyclonal. When the primary antibodies were monoclonal, peroxidase-conjugated, rabbit anti-mouse IgK (Kirkegaard and Perry Labs, Gaithersburg, MD) was used as the secondary antibody.

#### *Phospholipase A and hemolytic assays*

Qualitative analysis for phospholipase A (PLA) activity was performed following the protocol of MARINETTI (1965). Hemolytic activity was assayed by using rat erythrocytes according to the method of BERNHEIMER *et al.* (1976).

## RESULTS

#### *Trimeresurus wagleri* crude venom lethal potency, proteolytic and hemolytic activity

Crude venom of *T. wagleri* possessed moderate proteolytic activity (35 units of trypsin activity per mg venom). Crude venom protein content was 62–65%. The murine i.p. LD<sub>50</sub> of crude venom pooled from three snakes was 4.36 mg/kg. Mice succumbing to the effects of crude venom showed a sudden onset of tachypnea, tremors and myoclonus which led to

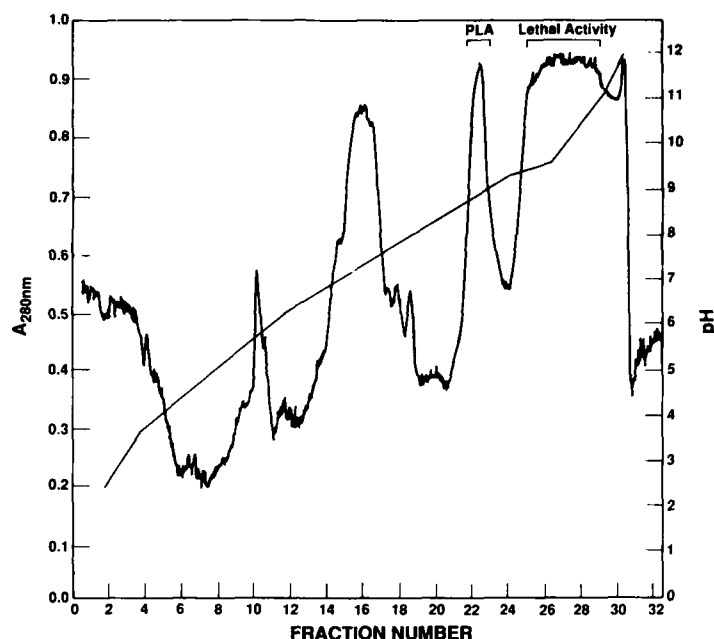


FIG. 1. ISOELECTRIC FOCUSING OF CRUDE VENOM OF *T. wagleri*.

Isoelectric focusing was performed in a 110 ml electrofocusing column. The gradient was prepared from a less dense solution consisting of 51 ml of water, 4 ml of 8% (w/v) ampholine (pH 3–10) and a more dense solution consisting of undialyzed sample, 8.5 ml of 8% (w/v) ampholine (pH 3–10) and 25 g of sucrose in a final volume of 55 ml. Focusing was carried out at 4°C for 24 hr with a final potential of 1000 V. Fraction volume was 4 ml.

rapid prostration and cardiorespiratory death. Exophthalmus was observed in all animals succumbing to lethal venom doses. Gross necropsy indicated some pulmonary congestion with no other apparent gross effects. The crude venom in concentrations up to 500 µg per ml lacked direct hemolytic activity.

#### *Isoelectric focusing of T. wagleri venom*

Isoelectric focusing of crude *T. wagleri* venom resulted in four major peaks (Fig. 1). Strong PLA activity was detected in fraction 24 ( $pI = 8.9$ ) while weak activity was found in fraction 21 ( $pI = 7.6$ ). Lethal activity was detected in fractions 26–30 ( $pI = 9.6$ – $9.9$ ).

#### *Fractionation of crude venom*

Chromatographic analysis attempted with cation and anion exchange or hydrophobic resin was unsuccessful in isolating the material responsible for lethal activity which consistently appeared as an unbound peak in the void volume. Figure 2 shows the profile obtained from Superose-12 (molecular sieve) analysis of *T. wagleri* venom. Approximately nine peaks were observed. Lethal activity was detected only in peak D (fractions 19–20). The lethal potency of peak D was 0.9 mg/kg, indicating a five-fold increase in specific activity. Necropsy of mice succumbing to the lethal pool revealed no difference from those which succumbed to the crude venom. The lethal pool contained low levels of proteolytic activity (17 units of trypsin activity per mg peak D protein).

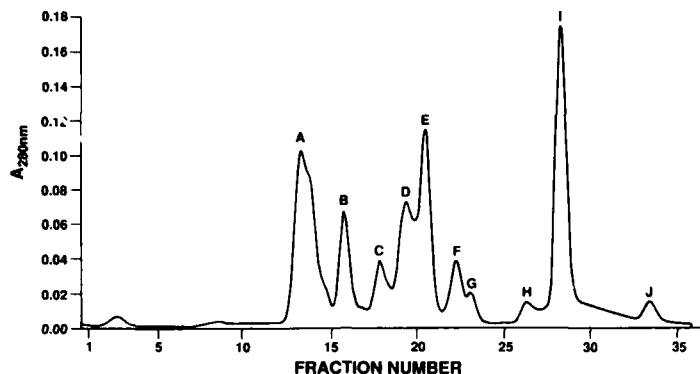


FIG. 2. MOLECULAR SIEVE CHROMATOGRAPHY OF CRUDE *T. wagleri* VENOM. Molecular sieve chromatography was performed at room temperature by using a Superose 12 HR10/30 column. Elution was carried out with Tris HCl 0.05 M, pH 7.2, containing 0.7 M NaCl. A flow rate of 0.5 ml/min was maintained and fractions of 1.0 ml were collected. All fractions were monitored for absorbance at 280 nm and lethal activity.

#### *Antigenic identity of Superose 12 lethal pool components*

Immunological survey by indirect ELISA of both crude *T. wagleri* and Superose-12 peak D indicated a lack of antigenic identity between *T. wagleri* venom toxins and  $\alpha$ -cobratoxin from *Naja n. kaouthia*, taipoxin from *Oxyuranus scutellatus*, crotoxin and crotamine from *C. durrisus terrificus*, and Mojave toxin from *C. scutulatus scutulatus*. A moderate reaction of identity was noted with polyclonal antibody against crude "type A" *Crotalus scutulatus scutulatus* venom. ELISA detection of extensive sharing of antigens among crotaline venoms has been noted by MINTON *et al.* (1984). Ouchterlony double immunodiffusion of peak D resulted in data identical to those obtained with ELISA. Reaction of the lethal pool with anti-*Crotalus scutulatus scutulatus* crude venom antiserum resulted in single precipitin band. Reaction of the lethal pool with anti-Mojave toxin antiserum was negative.

#### *Phospholipase A activity of Superose-12 peak D*

A qualitative assay for phospholipase A activity (MARINETTI, 1965) detected weak PLA activity in the Superose 12 lethal pool.

#### *Reverse phase chromatography*

Fractions 19 and 20 (superose-12 peak D) were individually purified further on a Bio-Rad C18 column, and the results are shown in Fig. 3. In both cases, toxicity was found only in peaks I and II. Moreover, peak I from fraction 19 (Fig. 3A) contained the same compound peak I from fraction 20 (Fig. 3B). The same was observed for peak II. Consequently, appropriate material from each reverse phase run was pooled for further study. Since peaks I and II were subsequently found to contain only one peptide each (see below), these are hereafter referred to as peptides I and II.

Multiple reverse phase runs were necessary to acquire sufficient purified material for subsequent experiments. Sample loads of at least 0.35 mg protein could be successfully chromatographed on the 0.46 cm diameter column. In some cases, a Bio-Rad 1.0  $\times$  25 cm reverse phase column was used, which could fractionate up to 1 mg protein per injection. In a typical preparation, about 0.28 mg peptide I and 0.44 mg peptide II were purified

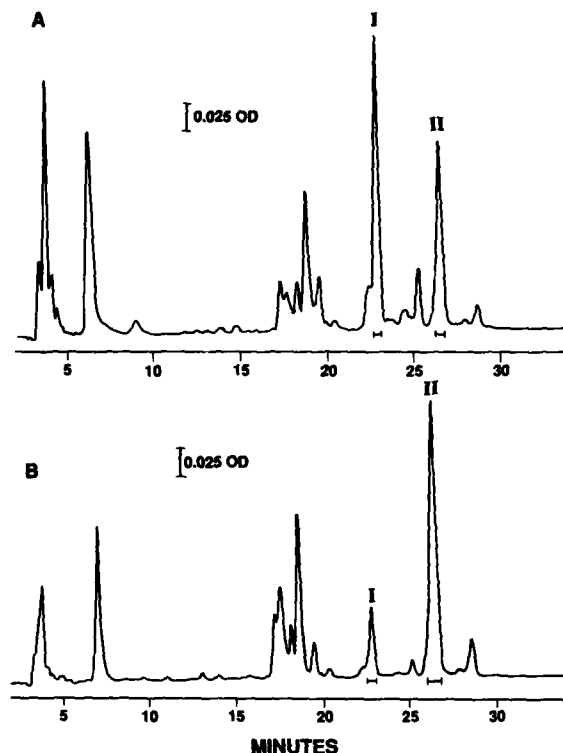


FIG. 3. REVERSE PHASE CHROMATOGRAPHY OF SUPEROSE-12 PEAK D, FRACTION 19 (PANEL A) AND FRACTION 20 (PANEL B).

The column was a Bio-Rad Hi-Pore RP-318,  $0.46 \times 25$  cm. Solvent A was 0.06% TFA and solvent B was 0.05% TFA/70% acetonitrile. Flow rate was 1.0 ml/min and the temperature was  $30^\circ\text{C}$ . The column was equilibrated with 12% B. After injection of sample, the solvent was held at this concentration for 3 min, followed by a linear gradient to 33% B at 30 min. The column was then eluted with 100% B for 7 min. Optical density (OD) of the column effluent was monitored at 210 nm. Portions of the eluents constituting peaks (peptides) I and II are indicated by solid bars.

from 18–20 mg crude venom. Peptide I had a murine i.p.  $\text{LD}_{50}$  of 0.369 mg/kg, while peptide II had a murine i.p.  $\text{LD}_{50}$  of 0.583 mg/kg. Both peptides elicited tachypnea, ocular proctosis, rapid collapse and spasms in mice.

#### *Thermostability of peptide I*

Lethal peptide I was markedly thermostable. The toxin retained 100% lethal potency after autoclaving at  $121^\circ\text{C}$  for 40 min. The onset and characteristics of symptoms were identical to unheated toxin.

#### *Amino acid sequencing of peptides I and II*

The primary structures of peptides I and II are shown in Fig. 4. In both cases, interpretable data abruptly ceased with residue 22, suggesting that complete sequences had been obtained. Further, inspections of the chromatograms showing the phenylthiohydantoin (PTH) derivatives obtained from each cycle of Edman degradation (data not shown) revealed the presence of only trace amounts of extraneous residues, suggesting

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
Peptide I:	G	G	K	P	D	L	R	P	C	H	P	P	C	H	Y	I	P	R	P	K	P	R
Peptide II:	G	G	K	P	D	L	R	P	C	Y	P	P	C	H	Y	I	P	R	P	K	P	R

FIG. 4. AMINO ACID SEQUENCES OF PEPTIDES I AND II.

high purity. The molecular weight of peptide I, based on sequence data, was 2504, while that of peptide II was 2530.

In both structures, residues 9 and 13 were identified as the PTH derivatives of S- $\beta$ -(4-pyridylethyl)-cysteine. Tests of the native toxins with Ellman's reagent (ELLMAN, 1959) indicated the absence of sulfhydryl groups. This result suggests that the toxins contain one intramolecular disulfide bond per mole, but the possibility of intermolecular disulfide bonds has not been eliminated.

#### Amino acid compositions

The relative amounts and ratios of amino acids in peptides I and II are summarized in Table 1. Residues per mole of each amino acid are in complete agreement with that calculated from the sequences, with the exception of S- $\beta$ -(4-pyridylethyl)-cysteine, which could not be quantitated because it was not eluted from the analyzer column. In addition, only those amino acids identified in the sequences were found in the hydrolysates. Thus it is extremely unlikely that an additional peptide was present and remained undetected in sequencing experiments due to a blocked N-terminus. No unusual peaks or amino sugars were observed on the amino acid analyzer chromatograms.

#### Extinction coefficients of peptides I and II

Absorbance spectra of peptides I and II (data not shown) demonstrated that each peptide had a maximum in the near ultraviolet, centered about 276 nm, with a shoulder at 282 nm. This indicated the presence of tyrosine and the absence of tryptophan (EDELHOCH, 1967), which is in agreement with sequencing data. Moreover, the ratio of absorbance at 276 nm to that at 260 nm (1.80) shows that no nucleotides are present. Therefore the molar extinction coefficient of peptide I at 276 nm was calculated as the sum of the contribution from tyrosine, 1280, plus that of the disulfide bond, 150, for a total of 1430. The result for peptide II, with two tyrosines and one disulfide per mole, was 2710.

TABLE 1. AMINO ACID ANALYSES OF PEPTIDES I AND II

Residue	Peptide I		Peptide II	
	Residues per mole	Nearest Integer	Residues per mole	Nearest Integer
D	1.18	1	1.03	1
P	6.77	7	6.58	7
G	2.12	2	1.73	2
C	n. d.*	n. d.*	n. d.*	n. d.*
I	1.00	1	0.92	1
L	1.12	1	1.00	1
Y	0.92	1	1.86	2
K	2.03	2	1.76	2
H	2.03	2	0.89	1
R	3.29	3	2.91	3

\* n. d. : not determined

These molar extinction coefficients were used to determine concentrations of solutions containing purified peptides I and II, since the BCA protein assay gave erroneously low results.

#### *Structural comparisons*

A search of the Swiss-Prot (University of Geneva, Switzerland) and Protein Identification Resource (National Biomedical Research Foundation) protein sequence databases, using computer programs from Intelligenetics Corp., Mountain View, CA, found no significant homologies with the sequences of peptides I or II. Considered with the results of the immunological survey, described previously, these data indicate that the structures of peptides I and II are unlike any heretofore reported.

#### DISCUSSION

SMITH and HINDLE (1931) described *T. wagleri* venom as neurotoxic. MINTON (1968) described a lack of rear limb paresis in mice injected with *T. wagleri* venom. TAN and TAN (1989) found that *T. wagleri* venom did not have a curaremimetic effect upon chick biventer cervicis nerve-muscle preparations. These workers also noted a lack of hemorrhagic and necrotizing activity.

The toxins isolated in the present study elicited tachypnea, exophthalmus, and myoclonus which led to rapid collapse in mice. The crude venom exhibited identical symptomology. Necropsy of animals succumbing after 12–20 hr to venom or toxin injections indicated a lack of gross hemorrhage or myonecrosis. The two isolated toxins were 22-residue peptides which had  $M_r$  of 2504 and 2530, respectively,  $pI$ s of 9.6–9.9, and both lacked phospholipase, proteolytic and hemolytic activity. The primary structures of both were determined by direct sequencing, which were corroborated by amino acid composition analyses and u.v. spectra. The sequences of peptides I and II were identical, with the exception of residue 10 (histidine in peptide I, tyrosine in peptide II). Peptides I and II had unusual compositions in that 7 of 22 residues were prolines. No sulfhydryl groups were found and the peptides probably contain one intramolecular disulfide bond per mole. Due to the agreement between the amino acid composition and direct sequence data, the presence of blocked polypeptides and/or carbohydrates is unlikely. The elution positions of the peptides from the size exclusion column suggest mol. wts of approximately 40,000. This apparently anomalous behavior is unexplained, but could result from aggregation or from non-covalent binding to other molecules. In contrast, elution of the toxins from the reverse phase column occurs at low acetonitrile concentrations, a characteristic of relatively low molecular weight peptides. However, we cannot yet rule out the possibility that the active principals are multimers formed by intermolecular disulfide bonds.

TAN and TAN (1989) reported the isolation of two toxins having  $M_r$  of 8900 and i.v. lethal potencies of 0.170 mg/kg and 0.190 mg/kg, respectively. The lethal potency of peptide II (0.583 mg/kg) in the present study had 62% of the toxicity of peptide I (0.369 mg/kg). These lethal potencies were determined using doses of toxin calculated by the molar extinction coefficient of each respective peptide. When protein concentrations were determined by BCA or Lowry, lethal potencies similar to those reported by TAN and TAN (1989) were obtained. Since only two toxins were present in the venom, we therefore conclude that peptides I and II correspond to the toxins isolated by TAN and TAN (1989).

The toxin caused symptomology suggestive of a hypotensive peptide or a neurotoxin. Hypotensive peptides such as those isolated from *C. viridis helleri* (DUBNOFF and RUSSELL, 1970; SCHAEFFER *et al.*, 1979) and *C. atrox* (BONILLA and RAMMEL, 1976) cause rapid



collapse, shock and respiratory arrest leading to death. In common with the *T. wagleri* toxins, a high proline content is a feature of these peptides, but sequence homology is not apparent. These toxins and several myotoxins (most notably myotoxin a; OWNBY *et al.*, 1976) have basic pIs in the range of 9–10 and are low mol. wt proteins (CAMERON and TU, 1977, 1978; OWNBY, 1982). Thus, the presence in snake venoms of low mol. wt, highly basic, proline-rich toxins is not unprecedented.

The unique morphological status among the genus *Trimeresurus* of *T. wagleri* was noted by BRATTSTROM (1964). MINTON (1974) commented on the distinct nature of *T. wagleri* venom compared with that of other *Trimeresurus* venoms. When compared with other *Trimeresurus* venom toxins, the lethal toxins of *T. wagleri* also appear unusual. SUGIHARA *et al.* (1983) described a lethal hemorrhagic toxin designated "mucrotoxin A" from the venom of *T. mucrosquamatus*. NIKAI *et al.* (1985) confirmed the hemorrhagic action of mucrotoxin A and isolated an additional hemorrhagic toxin ("mucrotoxin b"). These hemorrhagins had  $M_r$  of 15,000 and 27,000, respectively. OMORI-SATO and SADAHIRO (1979) resolved a lethal hemorrhagin of *T. flavoviridis* into two 60,000 mol. wt components. Most recently, SEKOGUCHI *et al.* (1989) characterized two lethal toxins (designated "TT-1" and "TT-2") from venom of *T. tokarensis*. Toxin TT-1 had an  $M_r$  of 71,000, a high content of aspartic acid and glutamic acid, and was hemorrhagic as well as proteolytic. Toxin TT-2 had an  $M_r$  of 25,400, high content of glutamic acid and serine, and was lethal. TT-2 was proteolytic, but not hemorrhagic. Both toxins were necrotizing and had  $LD_{50}$  values of 6.6  $\mu\text{g/g}$  and 7.2  $\mu\text{g/g}$ , respectively. These toxins required divalent cations ( $\text{Ca}^{2+}$  and  $\text{Zn}^{2+}$ ) for activity and were thermolabile. From these data, it is clear that *T. wagleri* venom toxins can be considered aberrant in their thermostability, lack of hemorrhagic activity, absence of metalloproteolytic lethal components and necrotic activity. The high lethal index of *T. wagleri* venom toxins appears distinct and could hypothetically be related to an absence of high concentrations of protease, which are significantly higher in other *Trimeresurus*. Most crotaline venoms with a high lethal index exhibit little, if any, proteolytic activity (GLENN *et al.*, 1983; MINTON and WEINSTEIN, 1984). The pI (9.6–9.9) of *T. wagleri* lethal toxins is compatible with the non-binding behavior observed upon toxin application to anion exchange and hydrophobic resin.

The amino acid sequences of both purified peptides when compared with a large number of toxin sequences were found to be unique. In addition, the semi-purified toxins lacked antigenic identity with a selection considered fairly representative of ophidian venom neurotoxins and myotoxins derived from elapine elapid and crotaline viperid venoms. Crude *T. wagleri* venom was found to contain at least one antigen in common with *Crotalus scutulatus scutulatus* crude venom. This antigen was not Mojave toxin. WEINSTEIN *et al.* (1985) found no immunoidentity between Mojave toxin and crude venom of *T. wagleri*. MINTON (1968) found that antiserum against venom of *T. flavoviridis* failed to neutralize *T. wagleri* venom, even though up to six antigens in *T. wagleri* venom were shared with *T. flavoviridis*, *T. steijneri* and *T. mucrosquamatus*. These data suggest that *T. wagleri* lethal venom components are antigenically distinctive.

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